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## Changes in Serum Complement Activity in Patients with Myasthenia Gravis.* (26050)

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It has frequently been suggested that the deficit in neuromuscular transmission characteristic of patients with myasthenia gravis might be produced by a curare-like agent which can circulate in the blood. In a search for such an agent, previously described(1), serum samples obtained from a series of individuals afflicted with myasthenia gravis were tested for neuromuscular blocking action. The assay involved measurement of the changes in muscle tension output of an indirectly stimulated frog sartorius muscle-sciatic nerve preparation which was immersed in the diluted serum.

Serum samples obtained from a few myasthenic patients caused a reduction in muscle tension output, and this result appeared to parallel the cytolytic destruction of fibers lying on the surface of the sartorius muscle used in the assay(1). From these and more recent observations(2), it is clear that serum exhibiting the above type of cytolytic activity occurs in 44% of patients afflicted with myasthenia gravis and in 22% of normal controls. The relative strengths of the active serums found in each group are unknown.

The results described above led us to think about the nature of the cytolytic system involved and the possible connection between the activity of this system and etiologic factors which operate in myasthenia gravis. As to the nature of the cytolytic system, we speculated that serum complement (C′) might be concerned because, as is well known, C′ plays an essential part in immune hemolysis. For this and other reasons, it seemed worthwhile to carry out determinations of C′ activity on serum obtained from myasthenic patients. Encouragement was provided by the early results which showed that in many myasthenic patients, serum C′ activity was far outside the normal range.

In this paper we have reported results of serum C′ analyses performed on samples collected from a large series of patients with myasthenia gravis. Many of these individuals were studied for long periods, with particular attention to changes in serum C′ activity and clinical condition. A brief survey

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of this work has been given previously (Nastuk *et al.*(3).

*Methods.* a) *Patients and controls.* The patients with myasthenia gravis who cooperated in this study showed considerable variation in severity of disease symptoms and in number and distribution of affected muscles. All patients were aided by adminstration of anticholinesterase drugs (usually pyridostigmine). The series of myasthenic patients includes cases in which a thymoma was present, in which therapy and treatment in addition to anticholinesterase drug administration were employed, and in which other clinical conditions of a possible complicating nature arose. Our rules for dealing with these situations are stated in the section under results concerned with clinical correlation.

Serum samples representing the normal control group were obtained from 4 females and 9 males all in good health and ranging in age from 20 to 50 years. From 2 of the females, samples were secured 1 to 4 days prior to beginning of menstruation and 1 to 2 days following cessation of menstruation.

b) *Blood sampling and storage.* At the start of this investigation, we specified withdrawal of medication from each myasthenic patient for a period of 12 hr prior to obtaining a blood sample. Adherence to this rule was difficult with severely afflicted patients who experienced distress if drug administration was stopped for more than 4 hr. Being forced therefore to draw blood samples at relatively short intervals following drug administration, we performed the following experiments to assure ourselves that the patient's serum C′ activity, as we determined it, was not influenced by his anticholinesterase intake.

Addition of pyridostigmine (10 mg/l) to serums obtained from a normal individual and a patient with myasthenia gravis had no effect on C′ activity assayed as described herein. This concentration of pyridostigmine is greater than that likely to be present in the serum of myasthenic patients receiving the drug.

In 5 myasthenic patients, C′ activities were determined on serum samples obtained before and during a 4 hour period following administration of pyridostigmine. No significant change in serum C′ activity was found.

A possibility not excluded by the preceding experiments is that prolonged administration of anticholinesterase medication causes slowly developed changes in serum C′ activity. We have no evidence bearing on the validity of this speculation, but even if correct, it does not provide a sufficient basis to explain our data. This point will be more fully treated in the discussion.

Details of blood sampling and storage procedure are as follows: without exercise or other special maneuvers, 10 to 20 ml of blood was drawn from the antecubital vein and placed in Pyrex centrifuge tubes which were covered with a sheet of Parafilm. After about 30 min., the clotted sample was centrifuged at 3000 rpm for 30 min., the serum was drawn off and placed in another Pyrex centrifuge tube and was tightly sealed with a sheet of Parafilm or, in later work, with a rubber stopper.

For 90% of the samples collected, the above procedures were completed within 3 hr whereupon, until removed for analysis, these serum samples were placed in a freezer and maintained at –35°C or lower. The remaining 10% of the samples, most of which were collected early in the study, received the same treatment except that they were stored one to 7 days at 0°C before final transfer to the freezer maintained at –35°C. Using serum from a normal individual, we were unable to detect a significant loss of C′ activity in an aliquot stored 7 days at 4°C. Therefore, we accept as valid C′ activities determined on those samples which were subjected to interim storage at 0°C.

c) *Analysis for serum complement activity* (4). 1. All glassware was carefully cleaned using a detergent solution (Alconox) followed by sulfuric acid-sodium dichromate solution, then was thoroughly rinsed and dried in an oven.

2—Stock sheep erythrocyte suspension. To 125 ml of a modified Alsever's solution (containing 2.45 g glucose, 2.2 g sodium citrate and 0.8 g citric acid per 125 ml solution) was added 375 ml of freshly drawn sheep's blood. After transport from the local source,

the treated blood was filtered through gauze into a flask which contained 2 x $10^6$ units of Penicillin G potassium, and 25 ml of disodium versenate solution. The latter solution was prepared by making up 0.15 M disodium versenate (disodium salt of ethylenediaminetetraacetic acid) and adding sufficient NaOH (1M) to raise the pH to 7.5. The thus preserved sheep erythrocyte suspension was stored in a stoppered flask at 4°C for 1 week before use, and was discarded after 5 weeks storage.

3. Veronal buffer. A stock solution was prepared by dissolving 5.75 g 5, 5-diethyl barbituric acid in 500 ml hot water. This was added to a solution containing 3.75 g sodium 5, 5-diethyl barbiturate and 85 g. NaCl and the mixture was diluted with water to 2 liters. For C′ determination, 50 ml of the above stock was mixed with 0.25 ml of 0.15 M $CaCl_2$, and 0.25 ml of 0.5 M $MgCl_2$. The mixture was diluted with water to 250 ml. The resultant buffer solution, called "veronal buffer" in the following text, was used for all subsequent washings and dilutions. It contains optimal concentrations of calcium and magnesium(5).

4. Standard red blood cell suspension. Approx. 20 ml of the thoroughly mixed stock sheep erythrocyte suspension was placed in a 40 ml centrifuge tube which was capped and spun at 3000 rpm for 5 min. The supernate was withdrawn and sufficient chilled (0°C) veronal buffer added to fill the tube which was then recapped, mixed and centrifuged as before. The washing procedure was repeated 5 times or more until the supernate was free of hemoglobin and cell fragments. A 7 ml aliquot of the lightly packed cell suspension was taken and mixed with 50 ml of chilled veronal buffer after which the mixture was filtered through a cotton plug and again chilled to 0°C. After thorough mixing of this suspension, a 1 ml sample was taken and diluted to 25 ml with water, which resulted in lysis of the erythrocytes.

Optical density of the lysate was determined at 541 mμ using a Beckmann DU spectrophotometer with square Pyrex cuvettes having a 10 mm light path, water serving as the blank. The O. D. obtained (always greater than 0.816) was used to calculate the amount of veronal buffer required to adjust the rbc suspension to the desired value of 2 x $10^9$ cells/ml. Following this procedure, a lysate produced from an rbc suspension containing 2 x $10^9$ cells/ml will have an O.D. of 0.816(4).

5. Sensitization of standard rbc suspension. The rabbit anti-sheep erythrocyte serum was appropriately diluted (see below) with veronal buffer (0°C). To the standard rbc suspension (at 0°C) an equal volume of the diluted anti-serum was added while mixing. The rbc suspension was then incubated for 30 min with shaking in a water bath at 37°C. The suspension now contained sensitized erythrocytes in a concentration of 1 x $10^9$ cells/ml. One ml of this mixture was diluted with water to 25 ml and optical density of the lysate was determined as before. Slight deviations from 0.408, the expected value, were taken into account in the final calculations.

The dilution factor for any particular lot of rabbit anti-serum was set such that the suspension of sensitized sheep erythrocytes, when prepared as described above, contained twice the concentration of antibody required for maximal sensitization of the cells. Determinations of extent of sensitization were carried out with normal serum whose C′ activity was close to the value representing the mean for a group of normal individuals.

6. Analysis for serum C′ activity-general procedure. All solutions and glassware were kept at 0°C unless otherwise indicated. To a 2 ml aliquot of each serum sample appropriately diluted with veronal buffer (see Sect. 7 and 8 below), 10 ml of standard sensitized erythrocyte suspension was added and mixed. Two ml aliquots of this mixture were added to each of 3 chilled test tubes which were then placed in a water bath at 37°C and shaken continuously. After 60 min incubation, one test tube was removed, quickly chilled to 0°C and centrifuged at 3000 rpm for 4 min. Without delay, most of the supernate was decanted into a clean tube, taking care to avoid transfer of erythrocytes. One ml of this decanted solution was pipetted into a clean tube to which 3 ml of water had been added. The

mixture was thoroughly stirred. After 90 and 120 min elapsed incubation time, the second and third tubes were removed from the water bath and similarly treated. Optical density of each final solution was determined as discussed earlier in connection with the standard rbc lysates.

For each serum dilution thus analyzed, optical density determined at various durations of incubation was plotted *vs.* time and from this plot an estimate was made of the O.D. which would be obtained at completion of the reaction. In practice, the slope of these plots was small and the estimated O.D. was usually very close to, if not equal to, the O.D. obtained for the sample incubated for 120 min. We therefore soon adopted the practice of incubating for 120 min. only, and considered that readings taken at this time represented the endpoint of the reaction. Final O.D. readings obtained by either method were corrected by subtracting the O.D. obtained in appropriate blank determinations in which the diluted serum was omitted.

7. Calibration of analysis for serum C′ activity. In this work, serum C′ activity was determined on the basis of degree of lysis produced in a standard suspension of sensitized sheep erythrocytes. Many analyses were carried out, over a long period. Therefore to maintain the internal consistency of our data, we required a calibration of each freshly prepared standard rbc suspension. The primary C′ standard used for calibration purposes was a large stock of serum obtained from a healthy human donor. Five ml aliquots of this serum were kept in rubber stoppered Pyrex tubes held at −35°C in the freezer containing all other serum samples.

Fig. 1 shows a typical calibration curve in which degree of lysis is plotted against a range of C′ concentrations (serial dilutions of the standard serum). Each time a group of serum samples was analyzed, a similar plot was prepared. The plots show that the 10-fold diluted serum standard produced, on different occasions, degrees of lysis ranging from 0.5 to 0.6, indicating that the various factors in the analysis were under reasonable control.

8. Determination of the relative C′ activity of individual serum samples. Each serum sample was diluted 10-fold with veronal buffer and the degree of lysis produced by an aliquot of this diluted serum was determined as outlined in Sect. 6. If the degree of lysis obtained did not fall on the central portion of the calibration curve obtained with the serum standard, the determination was repeated using an appropriately adjusted dilution of the serum sample. In practice, the dilutions required ranged from 0 to 30-fold.

Mean normal value of serum C′ activity was obtained by averaging C′ activities of 29 control samples obtained from 13 normal individuals (see results). Our standard normal serum which was used to construct the calibration curves had a C′ activity equal to this mean normal value, therefore the curve could be used to calculate directly the C′ activity of a particular serum, relative to mean normal C′ activity. We were fortunate in our choice of the normal serum standard; however any normal serum could have been used after determination of the appropriate scaling factor.

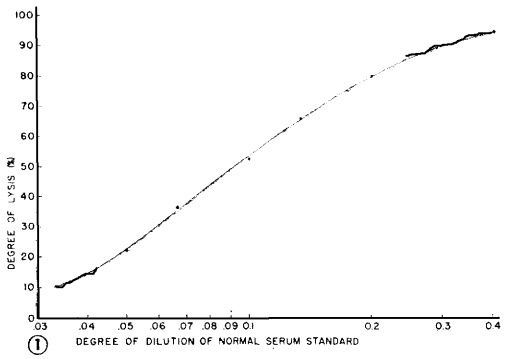
A simple method of dealing with the problems of standardization and determination of relative C′ activity might be to pool serum samples obtained from a large group of normal individuals and to use this pool as a source of standard serum C′ having unit activity.

*Results.* 1. Control series. Serum C′ activity was determined for 32 samples obtained from 13 normal persons (9 males, 4 females) whose ages ranged from 20 to 50 yrs. Average C′ activities for males and females were not significantly different. Tests on 2 of the females showed that menstruation does not affect serum C′ activity. For all 32 serum samples, C′ activities showed a standard deviation of the mean of ± 2%, and a *maximum* deviation from the mean of ± 19%.

2. Patients with myasthenia gravis. Serum C′ activities were determined for 68 patients with myasthenia gravis. In 34 cases, 3 to 14 serial determinations were carried out over a time span ranging from 2 to 44 months. In 12 cases, 2 C′ determinations were made over a time span ranging from 1 week to 9 months. In the remaining 22 cases a single C′ determination was made.

Serum C′ activities determined for these

patients were distributed over a wide range. Frequently values were obtained which were below or above the *extremes* of the range of our control series (lower limit = 0.8, upper limit = 1.2 mean C′ activity = 1.0). Forty of the myasthenic patients gave one or more serum samples whose C′ activity was below the lower limit of the control range, and values as low as 0.05 were found. Thirty-one patients gave one or more serum samples which showed C′ activity lying above the upper limit of the controls, the maximum value being 2.57. Evidence for wide variation in serum C′ activity was obtained in 17 patients who at different times showed values below and above the limit of the control range. Fifty-four of the 68 patients at some time showed a C′ activity lying beyond the limits of the control range (Fig. 2).



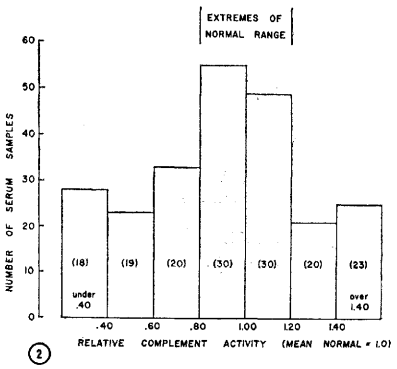




FIG. 1. Typical calibration curve showing relation between degree of lysis of a sensitized sheep erythrocyte suspension and complement activity expressed in terms of degree of dilution of normal serum standard.

FIG. 2. Showing distribution of C′ activities in serum samples obtained from 68 patients with myasthenia gravis. Numerals in parenthesis give No. of patients contributing serum samples whose C′ activities fell in the range indicated.

*Clinical correlation.* We have surveyed the case histories of the 68 myasthenic patients with special attention to the 3-6 month periods preceding and following the dates on which blood samples were taken. In particular we have tried to determine whether the patient's disease symptoms were increasing in severity or distribution (indicative of an exacerbation), diminishing in severity or distribution (remission) or constant (status quo). The criteria used in setting these 3 categories were: a—substantial changes in anticholinesterase drug intake; b—changes in the patient's work performance and living habits; c—changes in distribution of affected muscles or in severity of disease symptoms in these parts.

Of the entire series of cases, 22 were not included in the assessment of clinical correlation for the following reasons: a—insufficient number of blood samples or insufficient clinical data to establish the disease trend (15 cases); b—X-irradiation to thymus during sampling period (2 cases); c—thymectomy during sampling period (2 cases); d—presence of other disease (3 cases). In the remaining 46 uncomplicated cases, evidence of disease exacerbation was seen in 15. Eleven showed serum C′ activities below the normal range, one fell in the normal range, and 3 were above the normal range.

Evidence of disease remission was seen in 21 of the 46 uncomplicated patients accompanied in 11 of these cases by a rise in C′ activity which reached the supernormal range, 6 showed a rise in C′ activity which reached the normal range, 4 showed no change or a drop in C′ activity.

Fourteen cases were rated as status quo, 6 of these showed C′ activities which were within the normal range and showed little variation. The 8 remaining cases included 3 pregnant women each of whom gave birth to an infant exhibiting neonatal myasthenia gravis. One of these women showed, during the last trimester, a rise in serum C′ activity to supernormal levels. Her symptoms remained essentially unchanged and there was a slight rise in anticholinesterase medication during the course of the pregnancy, a change considered to be not significant. The second

woman showed during pregnancy a serum C′ activity so low that it could not be accurately determined. The third mother showed variable serum C′ activity with all values lying in the subnormal range.

The remaining 5 cases of the status quo group include the following: one patient whose C′ activity was fairly constant but subnormal, one patient whose C′ activity varied between the subnormal and normal range, one patient whose C′ activity showed a marked drop into the low subnormal range, one patient whose C′ activity rose from the high normal to the supernormal range, and one patient in whom the C′ activity remained fairly steady but in the supernormal range.

*Discussion.* The results reported here show that in patients with myasthenia gravis, serum complement (C′) activity fluctuates over an abnormally wide range. The clinical data indicate that these changes in serum C′ activity are related with exacerbation and remission of the disease.

Our findings direct attention to the possibility that an immune mechanism may play an etiological role in myasthenia gravis. Such an etiological basis would be greatly strengthened if it could be shown that the variations in serum C′ activity reflect the participation of C′ in a mechanism capable of producing the defects seen in myasthenia gravis.

An alteration in serum C′ activity may be produced in a variety of ways such as: (a) change in rate of synthesis, breakdown and excretion of C′, (b) shift in equilibrium between C′ stores and circulating C′, (c) binding of C′ by tissues, (d) disproportionation of C′ components, (e) production of C′ inhibitors. Clearly one might take the position that the changes in serum C′ activity in patients with myasthenia gravis are simply incidental accompaniments of the disease. In such a case, serum C′ changes would have no critical etiological significance but might nonetheless have great practical value in indicating the periods during which the disease process is active. It must be kept in mind that logically speaking, one *need not* consider the etiological process to be active merely because a patient shows a neuromuscular transmission deficit and a positive response to anticholinesterase drugs. On the contrary, there seems to be some reasonable ground for the argument that in many myasthenic patients, the neuromuscular transmission deficit long outlasts the period of activity of the defect-producing agents.

This view is not new. Keynes(6), reporting the effects of thymectomy in a group of myasthenic patients, states that the least postoperative improvement was obtained in those in whom the disease symptoms had been present for the longest periods. Keynes speculated that in longstanding chronic cases there may be present appreciable irreversible damage which sets a limit on the extent of the patient's postoperative remission. In further support of his idea, we may add that our study includes certain myasthenic patients who for long periods of time showed both an unchanging clinical status and a serum C′ activity always within the normal range. Tentatively, we have assumed that the disease process was inactive in these individuals.

One may next take up the point that the changes in C′ activity in myasthenic patients are caused by administration of anticholinesterase drugs. Evidence presented in the methods section indicates that pyridostigmine medication has no short-term influence on serum C′ activity. The drug may also have no long-term influence since certain of our patients showed a rise in serum C′ activity during its prolonged administration. Even more convincing is the fact that we have found subnormal C′ activity in serum of myasthenic patients who had not before received anticholinesterase medication. Our present evidence concerning this point is meager and we hope to extend it.

During early phases of this work we were attracted to the idea that the changes in serum C′ might result from its participation in an auto-immune reaction. The mechanism we visualized was the following. The myasthenic patient is one who has developed an auto-immunity against one of the components (M) of his skeletal muscle fibers. M, an entity found in the plasma membrane or intracellular elements was assumed to have combined with a foreign antigen S and the

combination MS, being antigenic, led to formation of auto-antibodies (A) against it. On reaching the muscle fiber, A would be bound forming MSA, a group which in turn would finally bind C′. Serum C′ would thus be consumed and in its presence there could be produced either a cytolytic destruction of the cell membrane or a subcytolytic alteration in its configuration. From the latter one might obtain a loss in the acetylcholine sensitivity of the postjunctional membrane, inhibition of muscle cell membrane conduction, failure of excitation-contraction coupling etc. Since the characteristic structures localized at the neuromuscular junction have a much increased membrane surface area, the above reaction might for this reason alone be intensified at this restricted site. We further assumed that depending on duration and severity of the above reaction, muscle fibers might be either reversibly or irreversibly damaged and that the terminal arbor of the motor nerve might be indirectly or directly involved.

This scheme has received strong support in explaining the causation of other disease conditions. For example, Roitt and Doniach(7) have presented an excellent summary of the evidence for the presence of auto-antibodies in thyroid disease. Their article provides a good source of the types of experimental attack which we believe must be made in the study of myasthenia gravis. Recent stimulating reviews concerning the participation of C′ in immune reactions are those of Lepow (8), and Osler *et al.* (9).

As is well known(8), serum C′ activity can change in many clinical conditions other than myasthenia gravis but this is of course no argument against its participation as outlined above in our scheme. What one needs is direct evidence concerning the presence and identity of the other components of our postulated system. Detection of the auto-antibody seemed the most critical, and our first attempt to do this was made possible by the kind cooperation of Dr. Seymour P. Halbert, Dept. of Ophthalmology, College of Physicians and Surgeons, Columbia University. Dr. Halbert used the Ouchterlony plate technic to test the reaction between serums obtained from our group of myasthenic patients and several possible sources of antigens including human skeletal muscle. The serums of certain myasthenic patients reacted faintly with the extract of human skeletal muscle but unfortunately the same result was also obtained with serums obtained from a few normal controls. This discouraging failure to obtain a positive result against extract of skeletal muscle might be explained on several grounds other than lack of antibody in the serum. We have since restudied the matter and are now attempting to demonstrate circulating auto-antibodies using the sensitive tanned hemagglutination technic. Very strong encouragement is provided by the evidence in the accompanying paper.

Our other line of evidence that changes in serum C′ activity may be the result of its uptake by an antigen-antibody complex is derived from the unpublished work of Plescia and his colleagues, who determined the activities of the individual C′ components in serums from myasthenic patients and found that $C'_1$ and $C'_3$ were normal, and $C'_2$, $C'_4$ were greatly reduced. This result is like that obtained in immune C′ fixation(8) but such interpretation may be complicated by presence of inhibitors of the C′ system which Plescia is able to detect.

Finally, in considering the hypothesis that myasthenia gravis is a disease involving a destructive auto-immune mechanism, one may note two additional points. Pathological changes in the skeletal muscle of myasthenic patients are recognized as important features of the disease(10). There is also convincing evidence that the thymus gland is capable of manufacturing antibodies(11), which opens the possibility that the connection between thymoma, thymic hyperplasia and myasthenia gravis is *via* an immune mechanism.

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## Immunofluorescence Demonstration of a Muscle Binding, Complement-Fixing Serum Globulin Fraction in Myasthenia Gravis.* (26051)

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Serum from some patients with myasthenia gravis has been shown to have a heat-labile cytolytic effect on sartorius muscle of the frog (1). Serum complement levels in many myasthenic patients in various stages of the disease often vary greatly beyond both the upper and lower limits of normal(2,3). These findings led to the speculation that some complement fixing immunologic system might be implicated in the pathogenesis of myasthenia gravis. In a test of this hypothesis, the immunofluorescence technic of Coons and Kaplan(4) was employed, using serum and skeletal muscle from patients with the disease.

We have demonstrated that the globulin fraction, prepared by 20% sodium sulfate precipitation of serums pooled from 10 myasthenic patients in the early progressive phase of their illness, as distinct from the normal serum globulin fraction prepared in like manner, has the following characteristics: 1. Ability to localize regularly and consistently in alternate striations of human skeletal muscle, both normal and myasthenic, as well as in skeletal muscle of one other mammalian species. This property was made evident by direct tagging of both myasthenic and normal globulins with fluorescein isothiocyanate. 2. Ability, when untagged, to block adherence of fluorescein tagged myasthenic globulin to skeletal muscle. 3. Ability, when once localized in skeletal muscle, to bind whole guinea pig complement. This fixation, *in vitro,* of heterologous complement was demonstrated by use of fluorescein conjugated rabbit antibodies to guinea pig complement. We have also shown that some individual whole myasthenic serums, including some of those utilized in the preparation of the myasthenic globulin pool, as distinct from normal human serums, are (1) capable of blocking adherence of fluorescein tagged myasthenic globulin to skeletal muscle striations, and (2) seemingly capable of fixing guinea pig complement in much the same way as does the myasthenic globulin fraction.

*Materials and methods. Source of serums, and preparation of globulin pools.* Serums from patients with myasthenia gravis were obtained on the wards and in the outpatient departments of Mount Sinai Hospital, and Columbia-Presbyterian Medical Center, New York. Diagnosis in each case had been established by history, physical findings, symptoms, and positive response to one or more anticholinesterase drugs. Control serums were taken from 12 medical house officers with an age and sex distribution comparable to that of the myasthenic patients. All were essentially healthy and denied histories of major system disease.

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